IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	
Maurice Israel et al.	Group Art Unit: 1649
Application No.: 10/049,296	Examiner: GREGORY S EMCH
Filed: August 6, 2002	Confirmation No.: 9468
For: PROCESS FOR IDENTIFYING MODULATING COMPOUNDS OF NEUROMEDIATORS	

DECLARATION BY INVENTOR UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Maurice Israël, hereby state as follows:

1. I am an inventor of the invention described and claimed in the above captioned application. From 1973 to 2005 I was employed by the assignee of the application, Centre National de la Recherche Scientifique (CNRS, France), as Director of Research and I was at the time of the invention Director of the Center for Cellular and Molecular Neurobiology. I am the main founder of Faust Pharmaceuticals, which is the exclusive licensee of the invention. A copy of my curriculum vitae and bibliography is attached hereto.

2. I have reviewed the Office Action mailed 5 January 2005 and the Office Action mailed 15 September 2005 with respect to the rejections of claims in the subject application under 35 U.S.C. §§ 102 and 103 as allegedly anticipated by, or obvious in view of, Reddy and Sastry (Brain Research, 168:287-98, 1979), and I have reviewed the cited document.

3. I have also reviewed claims 46-56 currently pending in the application, which have been rejected. Reddy and Sastry do not describe or suggest a process of making a preparation of calibrated pieces of mammalian cerebral tissue as described in the claims.

4. Reddy and Sastry describe passing minced brain tissue in Krebs-Ringer bicarbonate solution ten times through nylon bolting cloth having mesh sizes of 433 μm, 264 μm, 130 μm, or 44 μm. *See,* Reddy and Sastry at 289. Having been repeatedly passed through the cloth, the suspension produced by the method that Reddy and Sastry describe could only contain pieces of tissue with dimensions no larger than the mesh size (see Table 1).

5. The largest possible size of tissue pieces made by the method of Reddy and Sastry can be estimated by assuming that passing the tissue through the cloth produces cubes with dimensions equal to the mesh size. The volume of a cube having as its dimension 433 μm on a side (the largest mesh size used by Reddy and Sastry) is $(0.433\ mm)^3 = 0.081\ mm^3$. More realistically, because brain tissue is soft, it would be understood that the pieces would not actually be geometric cubes. The pieces of soft tissue would have rounded edges and corners so that the maximum volume of the resulting pieces may be estimated by modeling the pieces as spheres. The volume of a sphere having a diameter as large as Reddy and Sastry's largest mesh size of 0.433 mm is given by the formula $4/3\ \pi\ r^3 = 0.042\ mm^3$ (see Table 1). This represents a geometrical upper limit that is significantly lower than the average size of the pieces made according to the presently claimed method.

6. However, it should be noted that, owing to shear forces, there is not a clear geometrical relationship between the mesh sizes and the size of the structure that is preserved when using the mesh sizes suggested by Reddy and Sastry. In my original procedure, described in Israël et al.: Biochem. J. 160, 113-115, 1976, using 200 μm mesh produced 2.5μm synaptosomes from Torpedo.

7. Reddy and Sastry suggest methods that make suspensions of much smaller pieces of tissue having different properties than the pieces of tissue produced according to the methods of claim 46 and claims that depend from claim 46, but Reddy and Sastry do not suggest making a preparation containing any larger pieces of tissue.

8. The methods described and claimed in the present application result in a preparation that provides substantial benefits over the suspension produced following the teaching of Reddy and Sastry, for at least the following reasons.

9. Passing brain tissue through a mesh below about 0.5 mm as described in the Reddy and Sastry reference, gives synaptosomes (i.e. nerve terminals). By contrast, using mesh above 1 mm produces "microcubes", which were never prepared before the invention described in the present application (see Table 1). The mesh-filtration procedure utilised by Reddy and Sastry in 1979, was previously described by myself in 1976 (Israël et al.: Biochem. J. 160, 113-115, 1976) but they omitted to refer to me. The aim of that procedure was to pinch-off nerve terminals (synaptosomes) with minimal damage by forcing the tissue through gradually smaller meshes. In my 1976 paper, I forced the tissue through meshes decreasing to 200μm and obtained synaptosomes of 2.5μm diameter from Torpedo. Reddy and Sastry repeated the procedure and added a further 130 μm mesh, because

brain synaptosomes are smaller than in Torpedo, e.g. 0.5μm diameter. In my original work the 130 μm mesh started to damage the larger synaptosomes.

10. In spite of the fact that these synaptosomes were more viable than those pinched-off by conventional homogenisation procedures (Potter or Dounce), they were less viable than slices obtained with a McIllwain chopper that kept the neuronal and glial networks intact.

11. I realised that in order to get viability, one had to do the contrary of what we were doing and that we had to avoid decreasing the mesh size. We had never before analysed the structures found in a brain suspension after only passing the suspension through a relatively large (1 mm) mesh.

12. Because brain is soft, the adequate mesh was found to be a rigid nylon 1 mm square. This is exactly the contrary of what I, and others, had been previously doing, since previously we were successively decreasing the mesh size with the aim of pinching-off nerve endings. In using decreasing mesh sizes, myself, or Reddy and Sastry, were loosing precisely what could be kept, the "microcubes" produced by the methods of the present invention that we thus earlier missed. Following our discovery, we could not only prepare massive amounts of brain tissue "microcubes", but also enrich them.

13. In order to calibrate the "microcubes" thus obtained, they can be suspended in large volumes, for example about 1 litre or more of physiological saline. The "microcubes" sediment, spontaneously forming a "powder" on the bottom of the flask. The suspension looks like snow falling in a Christmas globe. Each "microcube" (about 0.5 mm on average) is a collection of many neurones glial cells and nerve terminals, connections are preserved. The "microcubes" are as viable as a slice, but unlike slices, "microcubes" can be aliquoted into comparable calibrated test samples.

14. The difference between the synaptosome preparation according to Israël et al. (1976) and Reddy & Sastry (1979) is the volume of the material isolated (see table 1). Reddy & Sastry preparation allows the isolation of material that is 10 times larger than the material produced according to Israël et al. (1976). At that time, we aim to isolate viable nerve terminals able to release a neurotransmitter. But isolating "microcubes" being larger (125 times compared to the '76 technique - see Table 1) and effective "neuronal networks" with a collection of viable neurones, glial cells and nerve terminals, is a real surprising invention which could not be obviously deducted from the above cited synaptosome preparation techniques. The "microcubes" after isolation are still able to release several neurotransmitters.

15. Thus, an advantage is that a suspension of calibrated "microcubes" can be aliquoted with a pipette into many tubes. One slice is never equal to another slice, while aliquots of, for example, 20 μl of a suspension of "microcubes" are comparable, permitting direct comparison of drug

effects. Screening drugs acting on neuronal interactions becomes possible, as it had never been before. The procedure is simple, but this had never been tried before. Until now, no other laboratory could measure in the supernatant above aliquots of "microcubes", the effect of a drug on 5 transmitters!

16. The mesh size is critical, below 1mm you start pinching nerve terminals; above you will get large fragments, viable like a slice. In the case of brain it seemed impossible that the organization would be made of a sum of functional units. Therefore, no one analyzed the suspension after a single, 1 mm filtration, in adequate experimental conditions. To our great surprise, it is like if the brain was made of a sum of small structures, i.e. the "microcubes", which were preserved by our procedure, because the density of their local connections made them slightly more solid than their surroundings. No one knew that such structures existed before, no one had ever tried to concentrate them and to purify them, as we did for the first time.

17. It is difficult to prove, and was not our aim to show that the "microcubes" represent structures that pre-exist in the brain, independently of the mechanical procedure, as if the brain was a sum of small ganglia. However the existence of barrels in mouse brain is recognized, or glomeruli in cerebellum. The denser the local connectivity is, the more solid the structure will be, which is dissociated from its environment. The fact is, with the claimed methods, we can collect a suspension of "microcubes", or "neurocubes", which is functional for hours, releasing a cocktail of transmitters. The claimed methods of making "microcubes" of brain tissue, and the benefits that can be obtained from them, were not described or appreciated by Reddy and Sastry, or anyone else in the field at the time the present application was filed.

18. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 15th May 2006

Maurice Israël

Table 1:

	Israël et al. 1976	**Reddy et al. 1979**	**Invention (Israël et al.)**
Mesh size (on a side)	200 μm	433 μm	1 mm
	0.2 mm	0.433 mm	1 mm
Area	0.040 mm^2	0.187 mm^2	1 mm^2
Volume (cube)	0.008 mm^3	0.081 mm^3	1 mm^3
Volume (sphere)	0.0041 mm^3	0.042 mm^3	0.52 mm^3
Nature of the material	**synaptosomes**		**neurocubes**

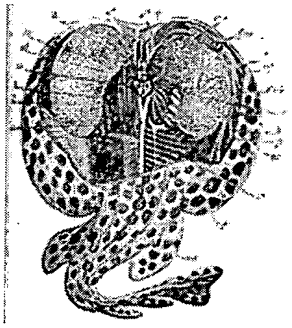
Maurice Israël

Maurice Israël, né à Alexandrie, a effectué ses études supérieures à Paris; il mène de front des études de médecine et de sciences, de la fin des années 50 au début des années 60. Mais c'est la recherche qu'il choisit et dès le début des années 60 il effectue son apprentissage du travail expérimental dans le laboratoire du Professeur R. Couteaux où il commence à s'intéresser aux jonctions neuromusculaires. Il y constate que, si la localisation de l'acétylcholinestérase dans les plis sous-neuraux postsynaptiques commence à être connue avec précision, il n'en est pas de même pour celles de l'acétylcholine et de son enzyme de synthèse la choline acétyltransférase, notamment à cause de l'insuffisance des méthodes de mesure.

Maurice Israël part donc , de 1963 à 1965, à Cambridge, à l'Institut de Physiologie animale dirigé par le Professeur Gaddum dans lequel il effectue ses premières recherches sur le fractionnement du tissu nerveux dans le laboratoire de V. P. Whittaker. Il y apprend à isoler les terminaisons nerveuses, les synaptosomes et à doser l'acétylcholine par son action sur le muscle dorsal de sangsue.

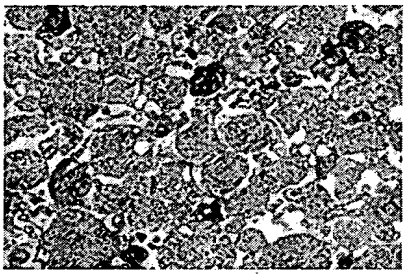
En 1967 , il part en Norvège, dans le laboratoire dirigé par le Dr. F. Fonnum pour participer à l'amélioration de la méthode de dosage chimique de la choline acétyltranférase qui fait intervenir la précipitation de l'acétylcholine par le tétraphénylborate.

En possession de ces trois techniques, d'abord au laboratoire de cytologie de la faculté des Sciences, rue Cuvier, puis au laboratoire de Cytologie, hôpital de la Salpêtrière, division Risler, Maurice Israël, démontre d'abord les localisations présynaptiques de la choline acétyltransférase et de l'acétylcholine au niveau des jonctions neuromusculaires. C'est à partir de l'organe électrique de torpille que M. Israël parvient à obtenir une fraction pure de vésicules synaptiques associée à un pic d'acétylcholine (140 n moles/g). C'était la confirmation biochimique de la théorie quantique de la libération de l'acétylcholine, l'hypothèse de la localisation du médiateur dans les vésicules ayant été formulée une quinzaine d'année avant et avait permis, entre autres, à Bernard Katz d'obtenir le prix Nobel en 1970.



Torpille, *cliquer pour agrandir*

Il était classique à l'époque (1970), de considérer que l'acétylcholine ne pouvait pas exister sous forme soluble dans le cytoplasme. Le fait que l'acétylcholine vésiculaire ne représente pas la totalité du médiateur, mais seulement 60 à 70 % du total était interprété comme une perte d'acétylcholine vésiculaire au cours de l'homogénéisation et l'observation que l'enzyme de synthèse, la choline acétyltransférase, ne soit pas localisée dans les vésicules apparaissait très surprenante. En collaboration avec Y. Dunant, M. Israël démontre la réalité du compartiment d'acétylcholine libre qui est le compartiment métaboliquement actif, utilisé et renouvelé au cours de stimulations physiologiques de l'organe électrique de torpille. C'est ce compartiment d'acétylcholine qui décroit alors que le compartiment d'acétylcholine vésiculaire n'est utilisé que dans des situations de stimulation intense.



Synaptosomes-organe électrique
de torpille. *Cliquer pour agrandir*

En 1973, l'équipe de Maurice Israël rejoint le laboratoire de Neurobiologie cellulaire de Gif sur Yvette dirigé par L. Tauc pour y constituer le département de Neurochimie. Plusieurs chercheurs du NBCM actuel l'accompagnent ou le rejoignent, N. Morel, F.-M. Meunier, Y. Morot-Gaudry, M.-F. Diebler, S. O'Regan. Parmi les résultats importants obtenus depuis, on peut citer :

- La libération d'ATP postsynaptique : une médiation rétrograde
- L'accumulation de calcium dans les vésicules synaptiques
- L'identification d'une protéine présynaptique, le médiatophore capable de libérer l'acétylcholine lorsqu'on l'intégre dans une membrane de liposomes.
- Clonage du transporteur vésiculaire d'acétylcholine
- Clonage d'un transporteur de choline

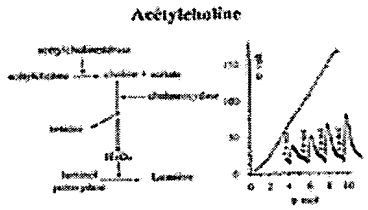
Cliquer pour agrandir

Maurice Israël, responsable du département de Neurochimie (1973) ou Directeur du laboratoire de Neurobiologie cellulaire et moléculaire (1994), n'en reste pas moins un

chercheur et avant tout un expérimentateur hors pair qui développe ses propres recherches :

Il poursuit ses investigations sur le médiatophore, une protéine de 15 kD et montre que, lorsqu'on le fait exprimer dans des cellules en culture, ces dernières s'avèrent capables de libérer l'acétylcholine dont on les a préalablement chargées. Ces résultats montrent que cette protéine peut assurer ou au moins participer à la libération de neuromédiateur.

Parallèlement à ces travaux sur le médiatophore, M. Israël a été l'auteur, au cours de cette dernière décennie, d'avancées technologiques importantes. Il a mis au point des méthodes de chemiluminescence qui permettent de mesurer plusieurs des neuromédiateurs importants du système nerveux. Ces méthodes consistent à dégrader le neuromédiateur par différentes enzymes de façon à obtenir des composés susceptibles, en bout de réaction, de réagir avec le luminol et d'émettre de la lumière, dont la quantité est proportionnelle à la quantité de neuromédiateur de départ. L' Acétylcholine a été la première à être dosée de cette manière par M. Israël :



Mise en évidence par chemiluminescence de la libération d'acétylcholine. *Cliquer pour agrandir*

Puis, sur le même principe, il réussit successivement le dosage du glutamate, du GABA et plus récemment de la dopamine. Ces méthodes, faciles à mettre en oeuvre et peu onéreuses ont été largement reprises et utilisées par l'ensemble de la communauté neurobiologique internationale.

Au cours des années pendant lesquelles il a assuré la direction du laboratoire, M. Israël s'est appliqué à développer, au sein du laboratoire, les collaborations entre chercheurs dont les thématiques ou des techniques pouvaient s'avérer complémentaires. C'est ainsi que tout récemment, il a eu l'idée et lancé un projet de recherche extrèmement important et ambitieux qui vise améliorer, *in fine*, l'état de malades atteints de la myopathie de Duchenne. En associant les spécialistes du laboratoire travaillant sur les cellules musculaires (S. De la Porte, E. Chaubourt) à ceux s'intéressant au monoxyde d'azote (G. Baux, P. Fossier, Y. Morot-Gaudry), il a suscité les travaux qui ont permis la réactivation de l'utrophine. Cette protéine embryonnaire pourrait remplacer la dystrophine, qui est manquante chez les malades atteints de myopathie de Duchenne et leur rendre la force musculaire qui leur fait défaut.

En décembre 2000, au milieu de son second mandat de 4 ans, M. Israël abandonne la direction du NBCM afin, d'une part, de susciter et favoriser l'évolution et la restructuration du NBCM et, d'autre part, de se ménager quelques années de recherche pour mettre à l'épreuve, au sein du NBCM, quelques unes des nombreuses idées issues

de la rencontre entre son importante culture physiologique et biochimique et sa large expérience de la neurobiologie.

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Isolation of Pure Cholinergic Nerve Endings from the Electric Organ of *Torpedo marmorata*

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A rapid method for the preparation of highly purified cholinergic nerve endings from the electric organ of *Torpedo* is described. The endings retain their cytoplasmic components, as shown by biochemical and morphological observations. The homogeneity of these synaptosomes make them a useful tool for further studies.

Whittaker (1959) and De Robertis *et al.* (1961) pioneered the isolation of nerve-ending particles from guinea-pig and rat cortex. The nerve endings become pinched off during homogenization, forming sealed synaptosomes that contain specific cytoplasmic markers, for example choline acetyltransferase in those derived from the cholinergic synapses. They also contain synaptic vesicles and sometimes a mitochondrion. The synaptosomes are able to respire, synthesize and release transmitter, take up precursors, and they also exhibit a membrane potential [for reviews, see Jones (1975) and Marchbanks (1975)]. They are therefore very useful for the study of pre-synaptic metabolism and mechanisms of transmitter release.

Unfortunately, the synaptosomal preparations at present available are obtained from various materials, such as rodent cortex, squid ganglia or *Octopus* brain, that are heterogeneous, since they contain nerve endings with different transmitters (see Jones, 1975).

We report here a method for the isolation of pure cholinergic nerve endings. The electric organ of *Torpedo marmorata* was chosen because of its homogeneity with respect to the transmitter acetylcholine and because of its numerous nerve terminals. Previous attempts with this tissue led only to damaged nerve-ending preparations (Israël & Gautron, 1969) and to the isolation of synaptic vesicles only (Israël, 1970; Israël *et al.*, 1968, 1970; Whittaker *et al.*, 1972).

Materials and Methods

The isolation procedure was rendered possible by the observation that chopping the electric organ preserves intact the innervated face of the electroplax (Morel, 1976). When other homogenization procedures are used, nerve endings get damaged.

Torpedoes were obtained from the Marine Station of Arcachon, France. A fragment of electric organ (20g) was finely chopped with a razor blade and suspended in 200ml of *Torpedo* physiological medium, which consists of 280mM-NaCl, 3mM-KCl, 1.8mM-$MgCl_2$, 3.4mM-$CaCl_2$, 5mM-$NaHCO_3$, 1.2mM-sodium phosphate buffer (pH6.8), 5.5mM-glucose, 300mM-urea and 100mM-sucrose. When equilibrated with O_2, its final pH is 7–7.2. All further steps were carried out at +4°C. After stirring for 30min, the suspension was forced through three stainless-steel grids mounted on syringes. The grids were purchased from Tripette et Renaud (Paris, France) and had square meshes of 1000, 500 and 200μm side, used in that order. The suspension was then filtered through a nylon gauze (square of 50μm side) under slight suction. The nylon cloth was washed with 50ml of physiological medium. The filtrate (fraction F) was then centrifuged at 6000*g* for 20min. The supernatant (fraction S) was discarded and the pellet (fraction P) resuspended in 20–25ml of physiological medium. Then 6ml was layered on to a discontinuous sucrose gradient (prepared 2h before). This gradient was composed, from bottom to top, of 8ml of physiological medium without urea but containing 0.7M-sucrose (final concentration), 8ml of physiological medium without urea but with 0.5M-sucrose (final concentration) and 12ml of physiological medium with 0.1M-urea and 0.3M-sucrose (final concentrations). It was centrifuged for 40min in an SW27 Beckman rotor at 63900*g* ($r_{av.}$ 11.8cm). The Beckman LS-65 centrifuge was set at its maximum acceleration rate, with its brake on. A tube slicer was used to collect the fractions, which were, from the top to the bottom of the tube, a clear supernatant (A), three bands at each interface (B, dense; C, wider; D, hazy) and a thick pellet (E).

Choline acetyltransferase (EC 2.3.1.6) was measured as described by Fonnum (1975). Acetylcho!-

esterase (EC 3.1.1.7) was measured by the method of Ellman *et al.* (1961). Acetylcholine was determined by the eserinized frog rectus technique as summarized by McIntosh & Perry (1950). Lactate dehydrogenase (EC 1.1.1.27) was determined as described by Johnson & Whittaker (1963), and proteins by the method of Lowry *et al.* (1951).

For morphological observations, fractions were half-diluted with the physiological medium and spun down (11000*g* for 20min). Pellets were fixed in 3% (w/v) glutaraldehyde in 0.5M-cacodylate buffer (pH7.4), post-fixed in 2% (w/v) OsO_4, dehydrated and embedded in Araldite.

Results and Discussion

Special attention was given for preparing a fraction of nerve endings of high purity and for maintaining intact their cytoplasmic content. Plate 1 shows a representative electron micrograph, at low magnification, of fraction C. Three independent experiments showed the same homogeneity. These endings contain numerous synaptic vesicles, glycogen granules, and, as in the intact tissue, few mitochondria are to be seen. The post-synaptic membrane does not remain adherent to the nerve endings. Table 1 shows that the fraction of pure nerve endings (C) contains the cytoplasmic marker lactate dehydrogenase, and a peak of acetylcholine and choline acetyltransferase clearly separated from the amounts found in fraction E. The pellet E, in which acetylcholinesterase is abundant, is heterogeneous and contains fragments of post-synaptic membranes with partially attached and damaged nerve endings, nuclei and erythrocytes.

The fact that 50% of the acetylcholine content of the filtrate (F) sediments in pellet P with only 25% of the choline acetyltransferase activity is expected, since the enzyme is present in the axoplasm of nerve branches (Israël, 1970). These are opened by the chopping and filtration procedures and account for about 50% of the total choline acetyltransferase activity.

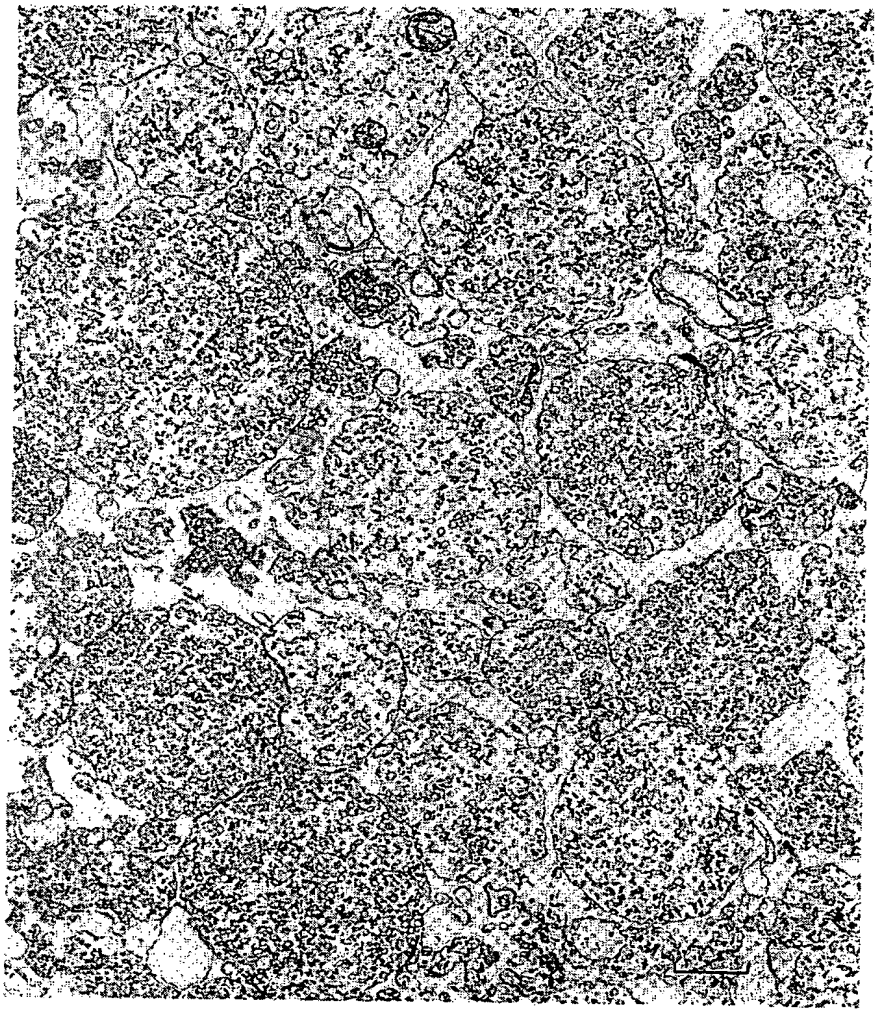
The ratio choline acetyltransferase/acetylcholine in the pure fraction C is higher than that in the pellet E, showing that the endings lost in pellet E have probably been damaged, loosing some cytoplasm. On the whole, we may calculate, by comparing the acetylcholine content of the filtrate (F) and fraction C, that the procedure purifies 20% of the intact nerve endings.

From electron-microscopic measurements it can be calculated that nerve endings represent about 11% of the electroplax volume; therefore we may attribute to them 1.66mg of the proteins present in the filtrate (F). If all the acetylcholine (271nmol/g) is occluded in these endings, a maximum specific activity of 163nmol of acetylcholine/mg of protein would be reached in a pure fraction. As the specific activity of

Table 1. *Isolation of cholinergic nerve endings: biochemical markers*

The results are means±S.E.M. of five experiments (three pooled gradients per experiment). Rf is recovery in primary fractions (S+P); Rg is recovery in gradients. For nomenclature of fractions, see the Materials and Methods section.

	Homogenate	F	S	P	A	B	C	D	E	Rf (%)	Rg (%)
Proteins (mg/g)	23.38±1.67	15.10±0.65	10.85±0.88	4.35±0.50	0.14±0.01	0.38±0.03	0.37±0.03	0.20±0.02	2.99±0.45	101	94
Acetylcholine (nmol/g)	485±49	271±17	79±12	150±5	2.1±0.1	13.1±1.7	57.2±4.8	15.1±2.0	72.1±5.4	84	106
Choline acetyltransferase (nmol/h per g)	2372±208	2137±315	1134±153	539±25	22±4	77±17	207±41	33±9	170±21	78	94
Acetylcholinesterase (mmol/h per g)	24.6±3.2	12.5±1.7	1.3±0.2	6.5±1.2	0.01±0.001	0.14±0.01	0.26±0.05	0.09±0.01	6.64±1.61	62	110
Lactate dehydrogenase (ΔE/min per g per ml)	44.7±14.9	33.2±13.8	21.3±10.2	12.0±0.9	0.19±0.11	1.46±0.18	2.68±0.32	0.97±0.19	6.23±1.03	100	96



EXPLANATION OF PLATE 1

Electron micrograph of the pure nerve endings (fraction C)

For details, see the text. The scale bar represents 1 μm.

(*facing p.* 114)

fraction C is 154 nmol of acetylcholine/mg of protein, we may consider that its purity is in accordance with the electron-microscopic observations.

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